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Purification and expansion of mouse invariant Natural Killer T cells for in vitro and in vivo studies

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TITLE:

Purification and expansion of mouse invariant Natural Killer T cells for in vitro and in vivo studies

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KEYWORDS:

iNKT cells; expansion; mouse; Vα14; CD1d; anti-CD3/CD28 beads; activation; lymphocytes

SUMMARY:

We describe a rapid and robust protocol to enrich invariant natural killer T (iNKT) cells from mouse spleen and expand them in vitro to suitable numbers for in vitro and in vivo studies.

ABSTRACT:

Invariant Natural Killer T (iNKT) cells are innate-like T Lymphocytes expressing a conserved semi-invariant T cell receptor (TCR) specific for self or microbial lipid antigens presented by the non-polymorphic MHC class I-related molecule CD1d. Preclinical and clinical studies support a role for iNKT cells in cancer, autoimmunity and infectious diseases. iNKT cells are very conserved throughout species and their investigation has been facilitated by mouse models, including CD1d-deficient or iNKT-deficient mice, and the possibility to unequivocally detect them in mice and men with CD1d tetramers or mAbs specific for the semi-invariant TCR. However, iNKT cells are rare and they need to be expanded to reach manageable numbers for any study. Because the generation of primary mouse iNKT cell line in vitro has proven difficult, we have set up a robust protocol to purify and expand splenic iNKT cells from the iVα14-Jα18 transgenic mice (iVα14Tg), in which iNKT cells are 30 times more frequent. We show here that primary splenic iVα14Tg iNKT cells can be enriched through an immunomagnetic separation process, yielding about 95-98% pure iNKT cells. The purified iNKT cells are stimulated by anti-CD3/CD28 beads plus IL-2 and IL-7, resulting in 30-fold expansion by day +14 of the culture with 85-99% purity. The expanded iNKT cells can be easily genetically manipulated, providing an

invaluable tool to dissect mechanisms of activation and function in vitro and, more importantly, also upon adoptive transfer in vivo.

INTRODUCTION:

Invariant Natural killer T cells (iNKT cells) are innate-like T lymphocytes that express a semi-invariant αβ T cell receptor (TCR), formed in mice by an invariant Vα14-Jα18 chain paired with a limited set of diverse Vβ chains[1], which is specific for lipid antigens presented by the MHC class I-related molecule CD1d[2]. iNKT cells undergo an agonist selection program resulting in the acquisition of an activated/innate effector phenotype already in the thymus, which occur through several maturation stages[3,4], producing a $CD4^+$ and a $CD4^-$ subsets. Through this program, iNKT cells acquire distinct T helper (T_H) effector phenotypes, namely T_H1 (iNKT1), T_H2 (iNKT2) and T_H17 (iNKT17), identifiable by the expression of the transcription factors T-bet, GATA3, PLZF, and RORγt, respectively[5]. iNKT cells recognize a range of microbial lipids but are also self-reactive against endogenous lipids that are upregulated in the context of pathological situation of cell stress and tissue damage, such as cancer and autoimmunity[2]. Upon activation, iNKT cells modulate the functions of other innate and adaptive immune effector cells via direct contact and cytokine production[2].

The investigations of iNKT cells have been facilitated by mouse models, including CD1d-deficient or Jα18-deficient mice, and by the production of antigen-loaded CD1d tetramers plus the generation of monoclonal antibodies (mAbs) specific for the human semi-invariant TCR. However, the generation of primary mouse iNKT cell line has proved difficult. To better characterize the antitumor functions of iNKT cells and to utilize them for adoptive cell therapy, we set up a protocol to purify and expand splenic iNKT cells of iVα14-Jα18 transgenic mice (iVα14Tg)[6], in which iNKT cells are 30 times more frequent than in wild type mice.

Expanded iNKT cells can be exploited for in vitro assays, and in vivo upon transfer back into mice. In this setting, for example, we have shown their potent anti-tumor effects[7]. Moreover, in vitro expanded iNKT cells are amenable to functional modification via gene transfer or editing prior to their injection in vivo[8], allowing insightful functional analysis of molecular pathways, as well as paving the way for advanced cell therapies.

PROTOCOL:

Procedures described here were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) (no. 1048) at the San Raffaele Scientific Institute.

NOTE: All the procedures must be performed under sterile conditions. All the reagents used are listed in the **Table of Materials**.

1. Spleen processing

1.1. Euthanize iVα14-Jα18 mice by inhalation of CO_2 according to the institutional policy.

NOTE: iVα14-Jα18 mice must be 8 weeks old or older. To avoid rejection of the cells from the in vivo transfer of the cells, consider that cells isolated from female mice can be adoptively transferred both in male and female recipients, whereas cells isolated from male mice can be transferred only in male recipients. For in vitro experiments, no gender bias must be considered. More mice can be pooled in order to obtain more cells.

1.2. Dissect the mouse spleen and smash it through a 70 nm cell strainer to obtain a single-cell suspension in 10 mL of phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS).

1.3. Centrifuge at 300 x g for 5 min.

1.4. Remove the supernatant by inversion and process with erythrocyte lysis buffer. Resuspend the cell pellet with 1 mL of sterile ACK (Ammonium-Chloride-Potassium) Lysing Buffer, incubate for 3 min at room temperature, and block with 5 mL of PBS with 2% FBS. Centrifuge at 300 x g for 5 min.

NOTE: ACK is commercially available; it consists of a solution of 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA (ethylenediaminetetraacetic acid) dissolved in bidistilled H_2O, pH 7.2-7.4. If homemade, sterilize by filtration with a 0.22 µm filter.

1.5. Remove the supernatant by inversion. Resuspend the cell pellet in 3 mL of PBS with 2% FBS and remove fat residues by pipetting. If needed, pool the cells coming from different mice.

1.6. Count the cells and keep 50 µL for FACS analysis.

2. T cell enrichment

NOTE: For the enrichment steps, work fast, keep the cells cold and use solutions pre-cooled at 4 °C overnight and then kept on ice

2.1. Centrifuge at 300 x g for 5 min.

2.2. Resuspend all the cells in the appropriate amount of PBS with 2% FBS (500 µL for 10^7 cells) and Fc blocker (2.5 µL x 10^7 cells); incubate for 15 min at room temperature (RT).

2.3. Wash with 1-2 mL of MACS separation buffer (MB) per 10^7 total cells and centrifuge at 300 x g for 10 min.

NOTE: MACS buffer is commercially available. It consists of PBS pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. If homemade, sterilize by filtration with a 0.22 µm filter.

2.4. Remove the supernatant by inversion and stain the cells with CD19-FITC and H2(IA^b)-FITC (use 5 µL x 10^7 cells in 100 µL of MB). Mix well and incubate for 15 min in the dark at 4-8 °C.

2.5. Wash cells by adding 1−2 mL of MB per 10^7 cells and centrifuge at 300 x g for 10 min.

2.6. Pipette off the supernatant completely and resuspend the cell pellet in 90 µL of MB per 10^7 total cells. Add 10 µL of Anti-FITC MicroBeads per 10^7 total cells. Mix well and incubate for 15 min in the dark at 4-8 °C.

2.7. Wash the cells by adding 1−2 mL of MB per 10^7 cells and centrifuge at 300 x g for 10 min.

2.8. Pipette off the supernatant completely and resuspend up to 1.25 x 10^8 cell in 500 µL of MB.

2.9. Place a LD column in the magnetic field of the MACS separator to proceed to the depletion. To avoid clogging, apply a pre-separation filter on the LD column and rinse with 2 mL of MB.

2.10. When the column reservoir is empty, apply the cell suspension onto the filter. Collect the unlabeled cells that pass through the column.

2.11. Wash 3 times with 1 mL of MB, only when the column reservoir is empty. Collect the total effluent, which will be enriched in T cells, and count the cells. Always keep 50 µL for FACS analysis.

3. iNKT cell enrichment

3.1. Centrifuge at 300 x g for 5 min and remove the supernatant by inversion.

3.2. Stain the cells with CD1d-tetramer-PE (mouse PBS57-Cd1d-tetramer), according to the antibody titration in 50 µL of MB per 10^6 cells. Mix well and incubate for 30 min in the dark on ice.

NOTE: The procedure can also be performed with APC-labelled mCD1d tetramers and anti-APC beads; adjusting the fluorochromes used in the following staining accordingly. In the present protocol we used mouse PBS-57-CD1d-tetramers provided by NIH. α-galactosylceramide (αGal-Cer) is the prototypical antigen recognized by iNKT cells; PBS-57 is an analogue of αGal-Cer with improved solubility[9]; the NIH Tetramer Facility provides PBS-57 ligand complexed to CD1d tetramers. However, other CD1d dimers/tetramers/dextramers are commercially available and can be loaded with a lipidic antigen as αGal-Cer. We envisage the possibility to adjust the protocol for their use.

3.3. Wash the cells by adding 1−2 mL of MB per 10^7 cells and centrifuge at 300 x g for 10 min.

3.4. Pipette off the supernatant completely and resuspend the cell pellet in 80 µL of MB per 10^7 total cells. Add 20 µL of Anti-PE MicroBeads per 10^7 total cells. Mix well and incubate for 15 min in the dark at 4-8 °C.

3.5. Wash cells by adding 1–2 mL of MB per 10^7 cells and centrifuge at 300 x g for 10 min.

3.6. Pipette off the supernatant completely and resuspend up to 10^8 cells in 500 µL of MB. Otherwise if cells exceed 10^8, adjust the volume accordingly.

3.7. According to the cell count, place a LS (up to 10^8) or MS (up to 10^7) column in the magnetic field of the MACS separator. Rinse the column with MB (3 mL for LS, 500 µL for MS).

3.8. Apply the cell suspension onto the column.

3.9. Collect unlabeled cells that pass through. Wash the column 3 times by adding the appropriate amount of MB (3 x 3 mL for LS column, 3 x 500 µL for MS column) only when the column reservoir is empty. The total effluent is the **negative fraction**.

3.10. Remove the column from the magnetic field and place it on a new collection tube.

3.11. Pipette MB onto the column (5 mL for LS column or 1 mL for MS column); push the provided plunger into the column and flush out the **positive fraction** (enriched in iNKT cells).

3.12. To further increase the iNKT cell recovery, centrifuge the negative fraction at 300 x g for 10 min and repeat steps 3.6-3.7 with a new LS or MS column. Pool the positive fractions and determine cell count. Keep 50 µL of both positive and negative fractions for FACS analysis.

3.13. Check the Purification steps by FACS analysis. Samples include: spleen ex-vivo, T cell enriched fraction, iNKT positive fraction and iNKT negative fraction. Stain the cells with: CD19-FITC, IAb-FITC, CD1d-tetramer -PE, TCRβ -APC and DAPI.

NOTE: The expected recovery from one iVα14-Jα18 mouse is $2x10^6$ iNKT cells .

4. iNKT cell activation and expansion

4.1. Activate purified iNKT cells with mouse T activator anti-CD3/CD28 magnetic beads in a 1:1 ratio.

4.1.1. Centrifuge the iNKT cell positive fraction at 300 x g for 5 min.

4.1.2. Meanwhile transfer the appropriate volume of anti-CD3/CD28 magnetic beads to a tube and add an equal volume of PBS, vortex for 5 seconds. Place the tube on a magnet for 1 minute and discard the supernatant.

4.1.3. Remove the tube from the magnet and resuspend the washed magnetic beads in the proper volume of complete RPMI to have 5 x 10^5 iNKT cells in 1 mL. Use this suspension to resuspend the centrifuged iNKT positive fraction.

4.2. Plate 1 mL of the cell suspension (5 x 10^5 iNKT cells) and anti-CD3/CD28 magnetic beads in a 48 well plate with 20 U/mL IL-2 and incubate at 37 °C.

4.3. After 5 days, add 10 ng/mL IL-7.

4.4. Split the cells 1:2 when they reach 80-90% confluence, always add 20U/mL IL-2 and 10 ng/mL IL-7. In these conditions, iNKT cells can be expanded for up to 15 days.

REPRESENTATIVE RESULTS:

The protocol described in this manuscript enable to enrich iNKT cells from the spleen of iVa14-Ja18 transgenic mice through an immunomagnetic separation process summarized in **Figure 1A**. Total spleen T cells are first negatively selected by depleting B cells and monocytes, followed by iNKT cell positive immunomagnetic sorting with PBS-57 lipid antigen loaded CD1d tetramers, that enable to specifically stain only iNKT cells. This protocol yields about 2 x 10^6 of 95-98% pure iNKT cells from the spleen of a single iVa14-Ja18 Tg mouse. No or really few iNKT cells can be detected in the negative fraction (**Figure 1B**).

After enrichment, iVa14 iNKT cells can be expanded with anti-CD3/CD28 beads plus IL-2 and IL-7 (**Figure 2A**), resulting in 30-fold expansion on average by day +14 of the culture as shown in **Figure 2B**.

Figure 3A shows the iNKT cell purity along with the expansion in vitro and the expression of the CD4 molecule. We observed a diminishment in the percentage of TCRβ^+ CD1d-tetramer$^+$ double positive cells: the strong activation with αCD3/αCD8 beads is inducing the downregulation of the iNKT cell TCR expression on the cell surface, and a double negative population is appearing. The majority of expanded iNKT cells were CD4$^-$. **Figure 3B** shows a characterization of the expression of lineage-specific transcription factors PLZF and RORγt on enriched iNKT cells at day 0 and 14 days after the expansion. This staining enables to identify the NKT1 (PLZF^{low} RORγt$^-$), NKT2 (PLZF^{high} RORγt$^-$), and NKT17 (PLZF^{int} RORγt$^+$) phenotypes. Being mostly NKT1 and NKT2, the enriched iNKT cells show a T_H0-like effector phenotype. This phenotype is conserved after 14 days of expansion as confirmed by the secretion of both IFNγ and IL4 after PMA/Ionomycin stimulation shown in **Figure 3C**.

FIGURE AND TABLE LEGENDS:

Figure 1: iNKT cell enrichment. **A**) Schematic representation of the immunomagnetic separation protocol. **B**) Flow cytometry analysis of each enrichment step. Percentage of T cell frequencies are shown in the upper plots, gated on viable lymphocytes. While percentage of iNKT cell frequencies along each step are shown in the lower plots, gated on viable CD19$^-$ TCRβ^+ lymphocytes. Staining on viable CD19$^-$ TCRβ^+ lymphocytes with unloaded CD1d tetramer allow to correctly draw the iNKT cell gate. One representative experiment is shown.

Figure 2: iNKT cell in vitro expansion. **A**) iNKT cell counts along iNKT cell expansion. One representative experiment is shown. **B**) Fold increase in iNKT cell number at day 7 and 14 after purification and activation. Means ± SD are shown.

Figure 3: Expanded iNKT cell characterization. A) Flow cytometry analysis of iNKT cell percentage and CD4 expression along the expansion period. Upper plots are gated on viable lymphocytes. Lower plots are gated on iNKT cells (viable CD1d-tetramer$^+$ TCRβ$^+$ lymphocytes). **B**) Phenotypic characterization of enriched (day 0) and expanded (day 14) iNKT cells. Plots are gated on iNKT cells (viable CD1d-tetramer$^+$ TCRβ$^+$ lymphocytes). Cells were intranuclearly stained for transcription factors with the Foxp3 Transcription Factor Staining Buffer Set. NKT1 (PLZF^{low} RORγt$^-$), NKT2 (PLZF^{high} RORγt$^–$), and NKT17 (PLZF^{int} RORγt$^+$) subsets were identified, frequencies of each subset are shown in percentage. **C**) Cytokine production by expanded iNKT cells at day 14. Plots are gated on iNKT cells (viable CD1d-tetramer$^+$ TCRβ$^+$ lymphocytes). Cells were stimulated for 4 hours with PMA 25 ng/mL/Ionomycin 1 μg/mL, in the presence for the last 2 hours of Brefeldin A 10 μg/mL. Cells were then fixed with PFA 2%, permeabilized with Permwash and then intracellularly stained for cytokine production. Gating strategy was set on the non-activated control, left panel. One representative experiment is shown.

DISCUSSION:

Here we show a reproducible and feasible protocol to obtain millions of ready-to-use iNKT cells. Due to the paucity of these cells in vivo, a method to expand them was highly needed. The protocol we propose requires neither a particular instrumentation nor a high number of mice. We exploited iVα14-Jα18 transgenic mice on purpose to reduce the number of mice needed for the procedure.

Another successful protocol for iNKT cell expansion from iVα14-Jα18 transgenic mice is available in the literature[10]. This protocol involves the generation, 6 days prior to iNKT cell purification, of fresh bone marrow-derived dendritic cells, then loaded with α-galactosylceramide and irradiated, plus IL-2 and IL-7. We consider the reduction of the number of the mice involved in the procedure a great advantage of the protocol. It is also time-sparing, since the setting up of the cell culture lasts a single day instead of a week. A possible limitation of the reproducibility of the current protocol could be the availability of iVα14-Jα18 transgenic mice, that are however commercially available. In absence of these mice, we envisage the possibility of using a large number of WT mice, but the protocol needs to be set up accordingly due to the paucity of iNKT cells in WT mice.

During the cell culture, we usually check the purity and the phenotype of expanded iNKT cells. The decrease in the percentage of TCRβ$^+$ CD1d-teramer$^+$ double positive cells (**Figure 3A**) can be explained by a natural downregulation of the invariant NKT cell TCR from the cell surface after activation. Moreover, the majority of expanded iNKT cells did not express CD4 (**Figure 3A**): this may represent an advantage in the context of an adoptive cell therapy, since CD4$^–$ iNKT cells were found to be the most effective in controlling tumor progression[11]. Moreover, the observed T_H0-like effector phenotype (**Figure 3C**) is entirely coherent with that observed in

human iNKT cells after in vitro expansion and restimulation[8,12-15]. The expanded cells are highly reactive in vivo and in vitro, thus useful in contexts of iNKT cell-based adoptive immunotherapies. Adoptive transfer of unmanipulated or expanded iNKT cells prevents or ameliorates acute Graft-Versus-Host Disease (aGVHD) leaving unaltered the Graft-Versus-Leukaemia effect[16-19]. Adoptively transferred human iNKT-cells expanded in vitro with αGalCer alleviate xenogeneic aGVHD and this effect is mediated by CD4- but not CD4+ cells[20]. Moreover, given that iNKT cells do not cause aGVHD, they constitute the ideal cells for CAR immunotherapy without need for deletion of their TCR and proved to have prolonged antitumor activity in vivo[8,15]. iNKT cells are currently exploited in on-going and concluded clinical trials[21-24].

In conclusion, the described protocol is fast, straightforward and allows a 30x increase in the number of iNKT cells recovered from a mouse spleen (**Figure 3B**). These cells can be easily exploited for in vitro recognition assays, co-culture systems, or adoptive cell therapy in preclinical studies. iNKT cells indeed, play a critical role in tumor immune surveillance, infectious diseases and autoimmunity. In these contexts, iNKT cells can represent a powerful tool, been an appealing alternative to conventional T cells devoid of the MHC restriction. The rapid generation of large amount of these cells and the possibility to further manipulate them in vitro can lead to the development of unprecedented therapeutical strategies.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Bendelac, A., Savage, P. B., Teyton, L. The biology of NKT cells. *Annual Review of Immunology.* **25** 297-336 (2007).

2 Brennan, P. J., Brigl, M., Brenner, M. B. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nature Reviews: Immunology.* **13** (2), 101-117 (2013).

3 Pellicci, D. G. et al. A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *Journal of Experimental Medicine.* **195** (7), 835-844 (2002).

4 Benlagha, K., Kyin, T., Beavis, A., Teyton, L., Bendelac, A. A thymic precursor to the NK T cell lineage. *Science.* **296** (5567), 553-555 (2002).

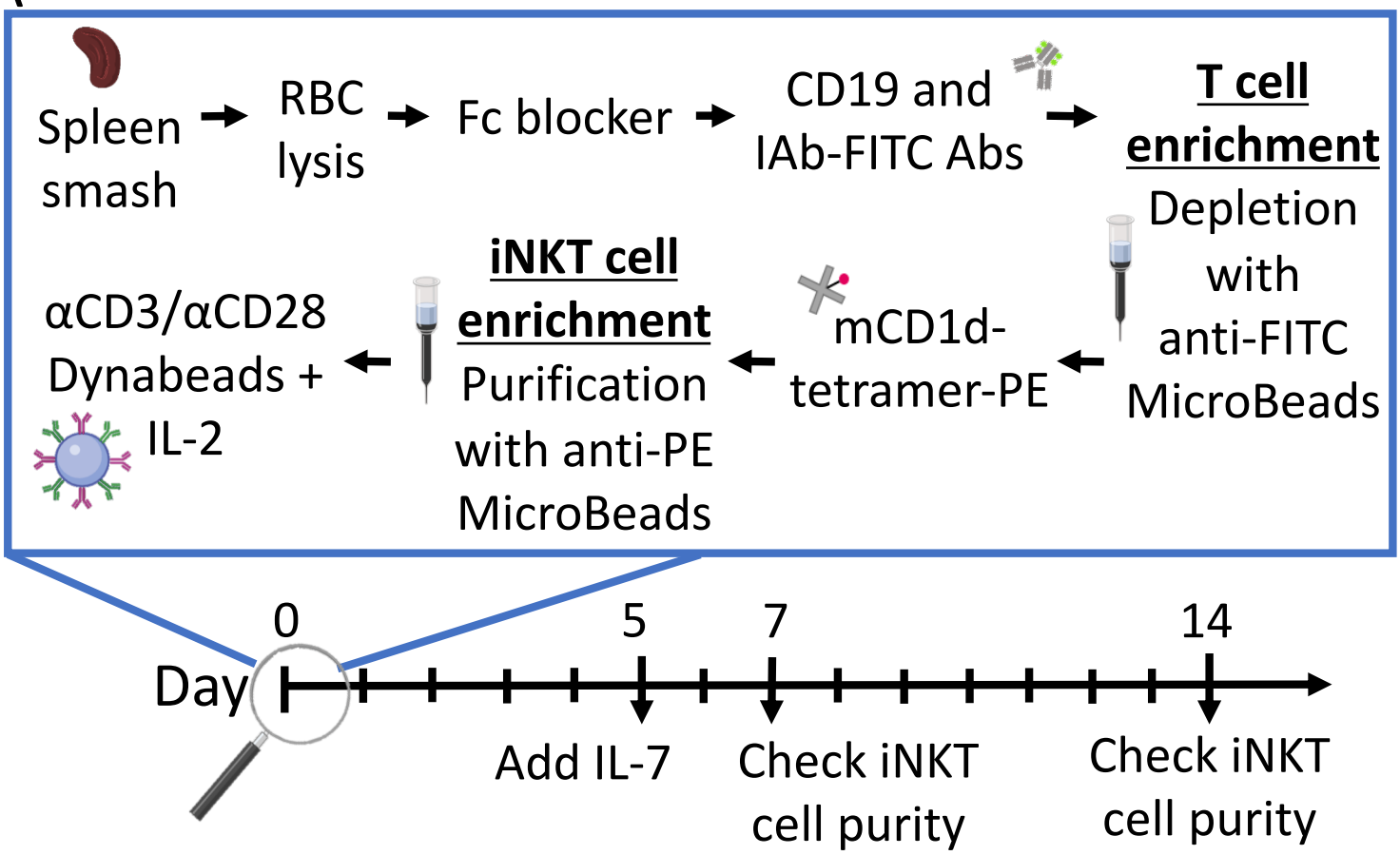
5 Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C., Hogquist, K. A. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature Immunology.* **14** (11), 1146-1154 (2013).

6 Griewank, K. et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors

control NKT cell lineage development. *Immunity.* **27** (5), 751-762 (2007).
7 Cortesi, F. et al. Bimodal CD40/Fas-Dependent Crosstalk between iNKT Cells and Tumor-Associated Macrophages Impairs Prostate Cancer Progression. *Cell Reports.* **22** (11), 3006-3020 (2018).
8 Heczey, A. et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood.* **124** (18), 2824-2833 (2014).
9 Liu, Y. et al. A modified alpha-galactosyl ceramide for staining and stimulating natural killer T cells. *Journal of Immunological Methods.* **312** (1-2), 34-39 (2006).
10 Chiba, A. et al. Rapid and reliable generation of invariant natural killer T-cell lines in vitro. *Immunology.* **128** (3), 324-333 (2009).
11 Crowe, N. Y. et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *Journal of Experimental Medicine.* **202** (9), 1279-1288 (2005).
12 de Lalla, C. et al. Production of profibrotic cytokines by invariant NKT cells characterizes cirrhosis progression in chronic viral hepatitis. *Journal of Immunology.* **173** (2), 1417-1425 (2004).
13 Tian, G. et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *Journal of Clinical Investigation.* **126** (6), 2341-2355 (2016).
14 Gaya, M. et al. Initiation of Antiviral B Cell Immunity Relies on Innate Signals from Spatially Positioned NKT Cells. *Cell.* **172** (3), 517-533 e520 (2018).
15 Rotolo, A. et al. Enhanced Anti-lymphoma Activity of CAR19-iNKT Cells Underpinned by Dual CD19 and CD1d Targeting. *Cancer Cell.* **34** (4), 596-610 e511 (2018).
16 Schneidawind, D. et al. Third-party CD4+ invariant natural killer T cells protect from murine GVHD lethality. *Blood.* **125** (22), 3491-3500 (2015).
17 Schneidawind, D. et al. CD4+ invariant natural killer T cells protect from murine GVHD lethality through expansion of donor CD4+CD25+FoxP3+ regulatory T cells. *Blood.* **124** (22), 3320-3328 (2014).
18 Schneidawind, D., Pierini, A., Negrin, R. S. Regulatory T cells and natural killer T cells for modulation of GVHD following allogeneic hematopoietic cell transplantation. *Blood.* **122** (18), 3116-3121 (2013).
19 Leveson-Gower, D. B. et al. Low doses of natural killer T cells provide protection from acute graft-versus-host disease via an IL-4-dependent mechanism. *Blood.* **117** (11), 3220-3229 (2011).
20 Coman, T. et al. Human CD4- invariant NKT lymphocytes regulate graft versus host disease. *Oncoimmunology.* **7** (11), e1470735 (2018).
21 Xu, X. et al. NKT Cells Coexpressing a GD2-Specific Chimeric Antigen Receptor and IL15 Show Enhanced In vivo Persistence and Antitumor Activity against Neuroblastoma. *Clinical Cancer Research.* **25** (23), 7126-7138 (2019).
22 Heczey, A. et al. Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. *Nature Medicine.* **26** (11), 1686-1690 (2020).
23 Exley, M. A. et al. Adoptive Transfer of Invariant NKT Cells as Immunotherapy for Advanced Melanoma: A Phase I Clinical Trial. *Clinical Cancer Research.* **23** (14), 3510-3519 (2017).
24 Wolf, B. J., Choi, J. E., Exley, M. A. Novel Approaches to Exploiting Invariant NKT Cells in Cancer Immunotherapy. *Frontiers in Immunology.* **9** 384 (2018).

(Figure 1)

A



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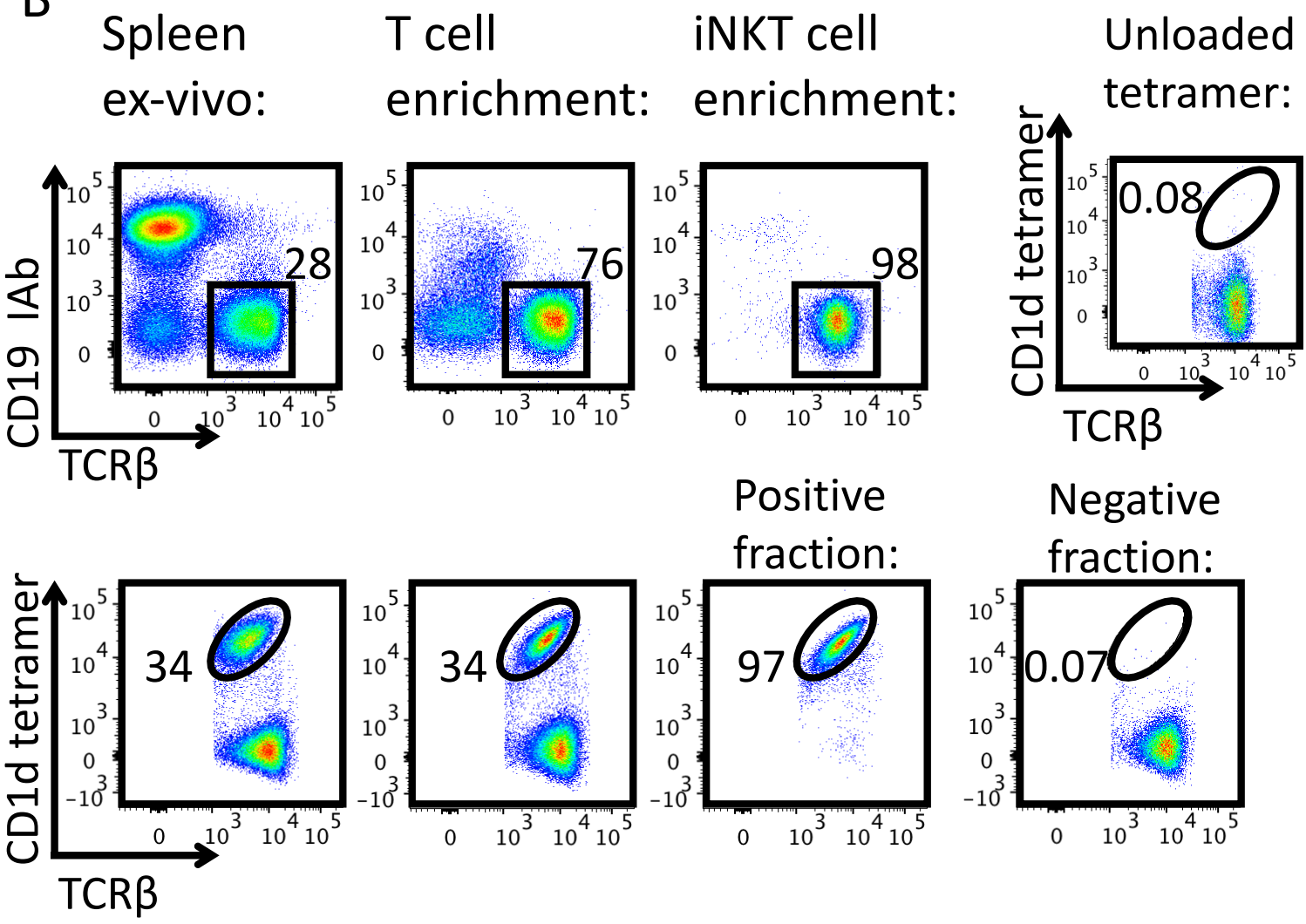

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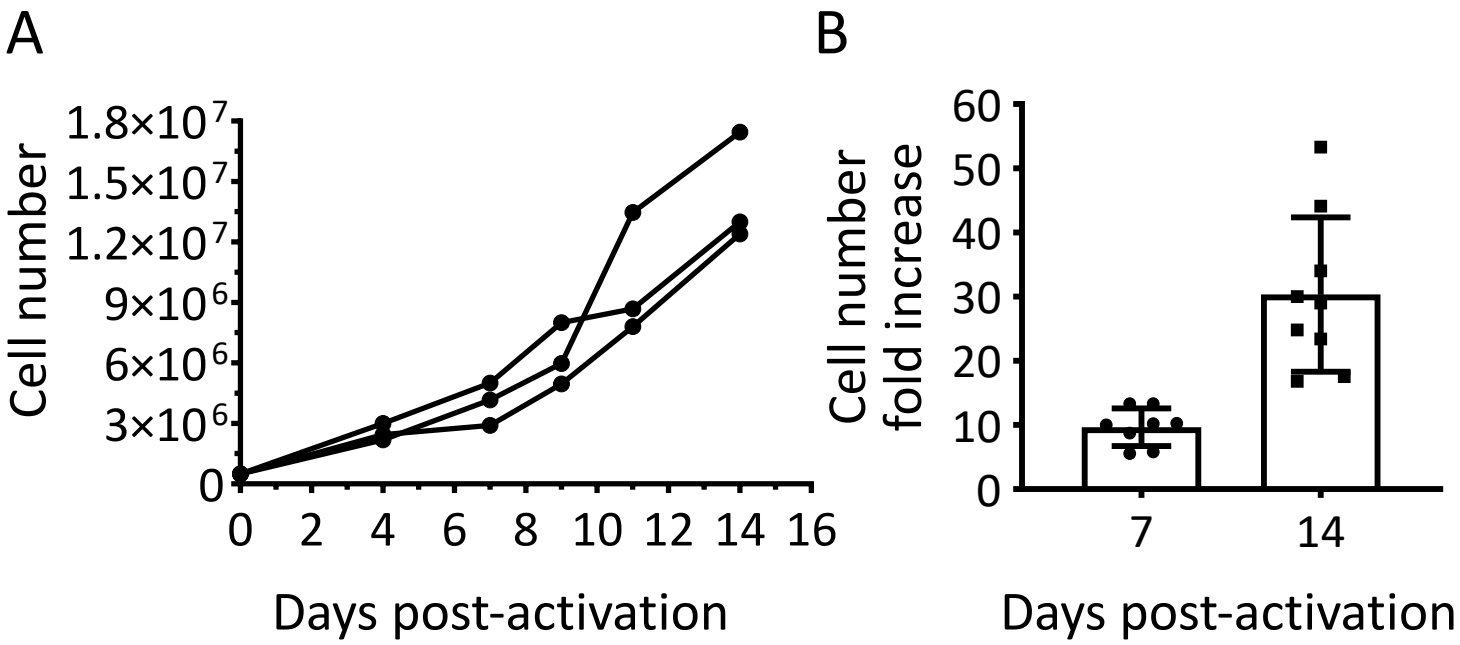

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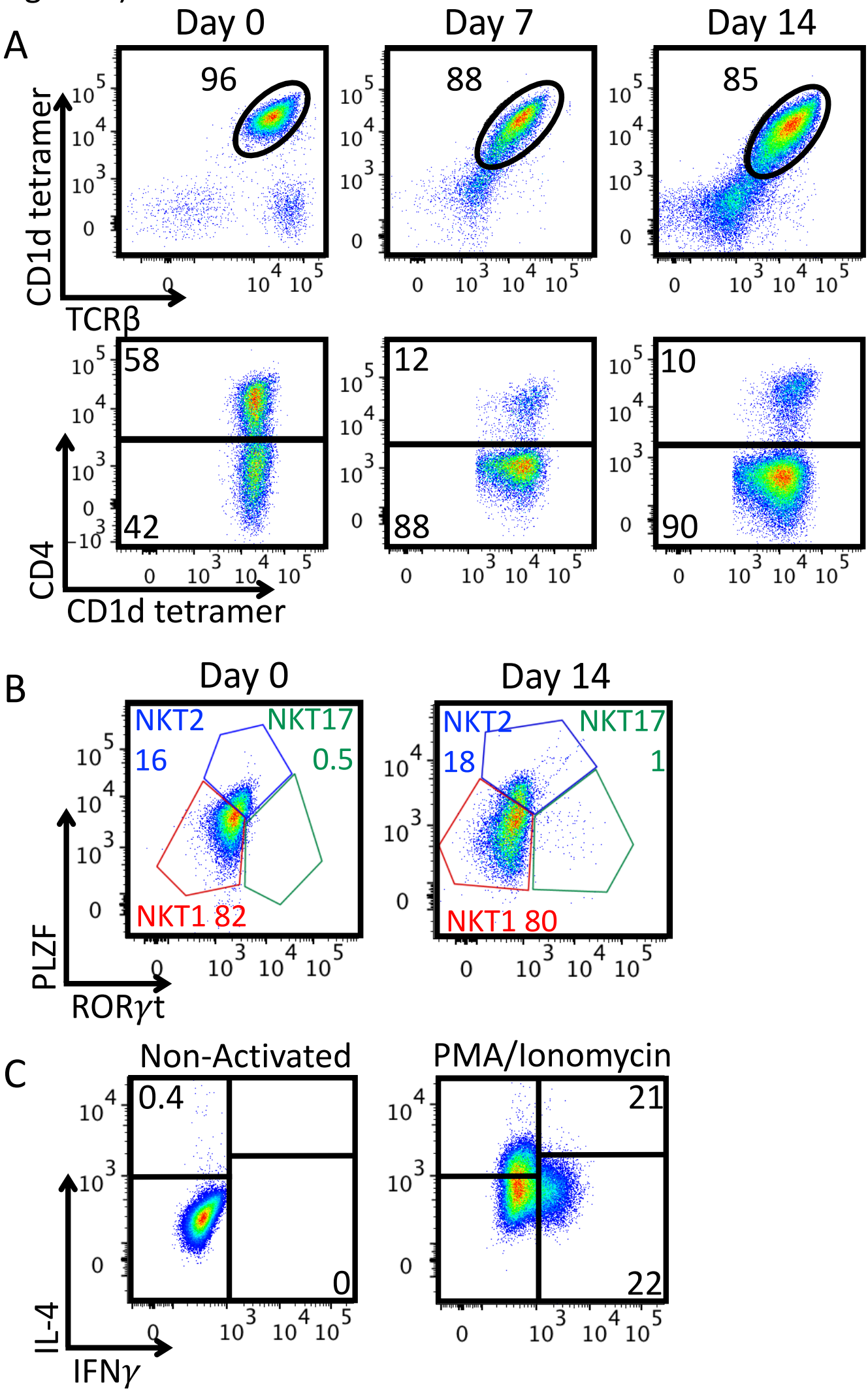


Name of Material/ Equipment	Company	Catalog Number
Ammonium-Chloride-Potassium (ACK) solution	in house	
anti-FITC Microbeads	Miltenyi Biotec	130-048-701
anti-PE Microbeads	Miltenyi Biotec	130-048-801
Brefeldin A	Sigma	B6542
CD19 -FITC	Biolegend	115506
CD1d-tetramer -PE	NIH tetramer core facility	
CD4 -PeCy7	Biolegend	100528
Fc blocker	BD Bioscience	553142
Fetal Bovine Serum (FBS)	Euroclone	ECS0186L
FOXP3 Transcription factor staining buffer	eBioscience	00-5523-00
H2 (IAb) -FITC	Biolegend	114406
hrIL-2	Chiron Corp	
Ionomycin	Sigma	I0634
LD Columns	Miltenyi Biotec	130-042-901
LS Columns	Miltenyi Biotec	130-042-401
MACS buffer (MB)	in house	
MS Columns	Miltenyi Biotec	130-042-201
Non-essential amino acids	Gibco	11140-035
Penicillin and streptomycin (Pen-Strep)	Lonza	15140-122
PermWash	BD Bioscience	51-2091KZ
PFA	Sigma	P6148
Phosphate buffered saline (PBS)	EuroClone	ECB4004L
PMA	Sigma	P1585
Pre-Separation Filters (30 µm)	Miltenyi Biotec	130-041-407
Recombinat Mouse IL-7	R&D System	407-ML-025
RPMI 1640 with glutamax	Gibco	61870-010
sodium pyruvate	Gibco	11360-039
TCRβ -APC	Biolegend	109212
αCD3CD28 mouse T activator Dynabeads	Gibco	11452D
β-mercaptoethanol	Gibco	31350010

	Comments/Description
0.15M NH4Cl, 10mM KHCO3, 0.1mM EDTA, pH 7.2-7.4	
clone 6D5 mouse PBS57-Cd1d-tetramers clone RM4-5	
heat-inactivated and filtered .22 before use	
clone AF6-120.1	
0.5% Bovine Serum Albumin (BSA; Sigma-Aldrich) and 2Mm EDTA	
clone H57-597	

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thanks, we did it.

2. Please define all abbreviations during the first-time use.

Thanks, we did it.

3. Please revise the following lines to avoid previously published work: 65-69, 156-161,171-173, 175-177.

Thank you for making us notice it, we fixed the indicated lines

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We added more details to the protocol steps

5. Step 1.1.: Please include how do you perform euthanasia in your experiment. Any age and sex specific bias?

Thanks, we specified these points after step 1.1

6. Please specify all volumes and concentrations used throughout. e.g., step 1.1 RPMI, step 1.2 erythrocyte lysis buffer, step 2.1, etc.

Thanks, we specified the volumes

7. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm)

Thanks, we did it

8. Line 115: Please provide the details of pre-cooling. How long and what temperature is used for pre-cooling?

Thanks, we did it before step 2.1

9. Line 117: Please include the number of cells

The number of cells cannot be indicated since it depends on the previous steps; we clarified to use all the cells obtained and to adjust the volumes of the solutions accordingly

10. Line 137: Please include how this is done.

Thanks, we did it

11. Line 171-173: Please provide more details on the washing steps, volume used, etc.

We added more details

12. Line 209: Please define the percentage of confluency.

Thanks, we did it

13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted the essential steps for the video

14. In the discussion section please also include some limitations of the protocol as well.

We added that the availability of iVα14-Jα18 transgenic mice could constitute a limitation to of the reproducibility of the protocol.

15. Figure 1: Please mention what the numbers represent (i.e., 0, 5, 7, 14) either in the figure or the Figure Legend. Please define whether the frequencies represented in Figure 1B and Figure 3 are in percentage.

Thank you for making us notice it, we added the definition of "day" in Figure 1. We specified that the frequencies in figure 1B and figure 3 are in percentage in the figure legend.

16. Please sort the table of materials in alphabetical order.

We did it

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the Manuscript by Delfanti et al, they describe how to isolate and expand mouse iNKT cells. The protocol is overall written in a way that it can be understood and replicated. The manuscript can be considered for publication, if following can be addressed:

Major Concerns:

- In representative FACS-plots, axis units must be shown.

We did it, however for the axis unit we couldn't use the recommended 20pt font otherwise the graphs would have been too big.

- In representative FACS-plots, the plots following expansion has been cropped (or there is a threshold

in FSC). This has to be shown in full. This protocol use CD3/CD28 expansion with contamination of T cells (Fig 1B), thus it is important to verify purity following expansion by showing graphs in full. Alternatively, consider an additional FACS purification step.

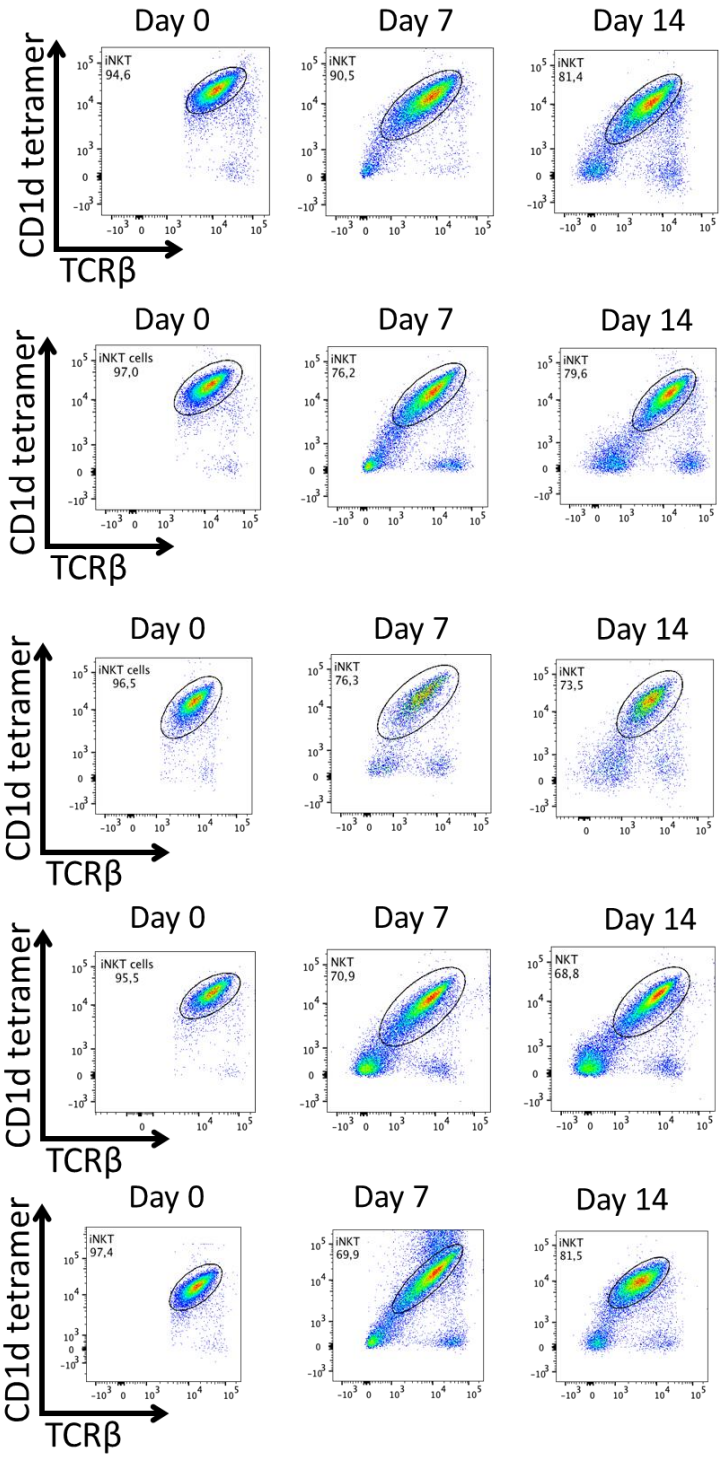
There was a gating on the TCRβ^+ but now we took it off, and the gating is directly on viable cells. We are aware that activation with CD3/CD8 beads could induce also expansion of contaminant T cell; however, very few TCRβ^+ CD1d tetramer$^-$ cells are present along the expansion (figure 3 A). Moreover, the strong activation with CD3/CD8 beads is inducing the downregulation of the iNKT cell TCR expression on the cell surface, and a double negative population is appearing (figure 3A)

- *Complete flow cytometric controls (w/wo PMA + technical controls (e.g. FMO or isotype control)) has to be shown in Figure 3 to justify gates drawn (especially 3C right).*

Thank you for letting us notice that the gating strategy was unclear.
The gates in figure 3C right panel were drawn on the non-activate control (without PMA/Ionomycin) shown in the left panel. We added the gating strategy in the figure legend for clarity.

- Flow cytometric confirmation should be shown for all expanded iNKTs as bar graphs (As for expansion in fig 2), or sufficient explanation should be provided.

We always check iNKT cell purity at day 0, 7 and 14 of culture and in figure 3A we showed one representative expansion. In figure here attached, you can see other 5 representative expansions with consistent percentages.



- Consider revising Figure 2 to show expansion curves for all experiments, merging A and B

We revised figure 2 plotting the curves of 3 experiments, previously shown as mean data. We can't show growth curves for all the expansions depicted in figure 2B, because we usually count the cells at day 7 and 14 post-activation, when we check iNKT cell purity. The experiments in figure 2A were made on purpose to have a cell growth curve.

Minor Concerns:
- In the method section, all units should be described in a reproducible way, e.g. RPM-->RCF

We fixed this

- Discussion should be up to date, with mouse and human iNKTs regarding clinically relevant phenotypes and how this expansion protocol is relevant. Also a balanced discussion of the functional impact of expansion should be extended.

We modified the discussion adding the clinical relevance of iNKT cells and the need for their expansion.

Reviewer #2:
Manuscript Summary:
The manuscript of Delfanti et al. addresses an interesting issue, i.e. the isolation and expansion of murine iNKT cells. This lymphocytic population is relatively rare and it is particularly difficult to perform ex-vivo experiments with them, as a consequence of their low abundance.
The scientific need is well explained, the protocol is generally clear and easy to follow and the discussion tackles the main topics of interest.
However, some minor corrections may improve the clarity of the manuscript.

Major Concerns:
Protocol:
_the majority of the descriptions within subchapters should be rendered less discursive and more protocol-like (i.e., within each description often 2/3 technical passages are present which should be subdivided)

Thanks, we separated steps into more sub-steps and tried to render them less discursive.

_although brands and descriptions of the reagents are present at the end of the manuscript, it should be better to write a minimal description of the reagents within the protocol (ie: ACK, MB)

We added the composition of the solutions also in the text.

_the "Comments and descriptions" at the end of the manuscript, that are intended to explain in house reagents compositions and antibody clones, does not have any recall in the main text. It should be added

Thanks for letting us noticing. At the beginning of the protocol, we added a statement recalling the provided Excel Table of Materials.

_Tetramer staining:
** it should be better specified that beside the one used in the protocol (provided by the NIH tetramer facility), other types of commercial tetramers/dextramers might be successfully implemented.*
** It should be better specified what PBS57 stands for and comment on the possibility that using other types of tetramers/dextramers the antigen might be called differently by other vendors (ie, classically aGalCer)*

We added a note after step 3.2 to explain what PBS-57 is and the existence of other commercially available CD1d multimers.

** a staining with the empty dimer should be added as control*

We added a staining with an unloaded tetramer in figure 1.

Minor Concerns:
Discussion:
It should be added more extensively for which purposes iNKT cells could be used, and thus why this protocol might be useful in those contexts.

We modified the discussion adding the clinical relevance of iNKT cells and the need for their expansion.